The influence of gender on 'tissue at risk' in acute stroke: an MRI DWI study in a rat model of focal cerebral ischaemia.

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Abstract

This is the first study to assess the influence of sex on the evolution of ischaemic injury and penumbra. Permanent middle cerebral artery occlusion (MCAO) was induced in male (n=9) and female (n=10) Sprague-Dawley rats. Diffusion-weighted imaging was acquired over 4h and infarct determined from T_2 images at 24h post-MCAO. Penumbra was determined retrospectively from serial ADC lesions and T_2-defined infarct. ADC lesion volume was significantly smaller in females from 0.5-4h post-MCAO as was infarct volume. Penumbral volume and its loss over time, was not significantly different despite the sex difference in acute and final lesion volumes.

Keywords: Acute stroke, Diffusion weighted MRI, Gender, MCAO

Introduction

The ischaemic penumbra is a well established concept in stroke, defined as a region of hypoperfused, metabolically active tissue surrounding the ischaemic core [1]. If blood flow is not restored, this tissue becomes incorporated into the ischaemic core and progresses to infarction [2, 3]. Therefore, it is crucial that the spatiotemporal evolution of ischaemic damage and penumbra is characterised in animal models of focal cerebral ischaemia to determine if there are differences in the optimal time for therapeutic intervention with regard to sex and in the presence of known stroke risk factors.

MR diffusion-weighted imaging (DWI) identifies tissue where diffusion is restricted as a result of cytotoxic oedema leading to a reduction in the apparent diffusion coefficient (ADC). Assessment of penumbral tissue can be determined retrospectively from the growth of the DWI or ADC lesion over time and the final infarct, a method used previously for both pre-clinical and clinical data sets [4].

In pre-clinical research, across the biological disciplines, there is still a considerable sex bias with studies on male animals outnumbering those in females and in particular this bias is most prominent in neuroscience research [5]. Reasons often cited for the prevalence of males over females are due to the concerns over contributions of the oestrus cycle, however a recent meta-analysis in female mice demonstrated that they were no more variable when tested throughout their oestrus cycle than males [6, 6]. Sex is a key factor influencing various aspects of human stroke including stroke outcome and response to treatment [7]. Likewise, in animal models of stroke, differences in molecular mechanisms of ischaemic cell death, stroke outcome and response to therapy have been observed between males and females [8]. To date, the acute spatiotemporal progression of ischaemic damage and fate of penumbra has not been characterised in female rats.

The study aims were: 1. to establish MRI diffusion thresholds of abnormality in male and female rats following permanent middle cerebral artery occlusion (MCAO); 2. Apply these thresholds to assess differences in the spatiotemporal evolution of ischaemic damage; 3. using retrospective analysis of ADC lesion and infarct volume to calculate penumbra, and compare penumbra volume and lifespan in males & females over the first 4h post-stroke.

Materials and Methods

All experiments were performed in age matched (12-16weeks) Sprague-Dawley rats (males: 300-350g, females: 250-280g , Harlan, UK) under licence from the UK Home Office, were subject to the Animals (Scientific Procedures) Act, 1986. The report was carried out in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive). Rats were randomly assigned to surgery using a random list generator (www.random.org).

Model of middle cerebral artery occlusion

Rats were intubated, ventilated and permanent MCAO carried out under isoflurane anaesthesia (5% induction, 2-2.5% maintenance in 70% N_2O 30% O_2). Body temperature maintained at 37°C and both femoral arteries were cannulated for mean arterial blood pressure (MABP) measurement and blood gas analysis. MABP and heart rate were continuously recorded under general anaesthesia, including scanning (AcqKnowledge, Biopac Systems, CA, USA). Permanent MCAO was induced with an intraluminal filament (diameter: 0.31-0.35mm, tip length: 5-6mm, Doccol, CA, USA) as previously described [9].

MRI scanning

MRI was performed on a Bruker Biospec 7-T/30-cm system with a gradient insert (121 mm ID, 400 mT/m) and a 72-mm birdcage resonator. A 4 channel phased array rat head surface coil was used for brain imaging. Arterial blood gas analysis was determined hourly (0-4h) during scanning.

Diffusion weighted imaging (DWI) was performed at 0.5h and hourly for 4hpost-MCAO to generate quantitative ADC maps and allow assessment of ischaemic injury.

At 24h post-MCAO, animals were re-anaesthetised and scanned with a RARE T_2 weighted sequence for assessment of infarct volume. For full details of imaging protocol see supplementary file.

Image analysis

Quantitative ADC maps, (x $10^{-3}mm^2/s$) generated from raw DWI images using Paravision 5 software (Bruker, Germany) were subsequently processed using Image J software (http://rsb.info.nih.gov/ij/).

Infarct volume was calculated by manually delineating the hyperintense region on T_2 slices which corresponded anatomically to the ADC slices acquired acutely. Area of infarct was

summed over all slices and multiplied by slice thickness to calculate total infarct volume which was corrected for brain swelling (Gerriets *et al*, 2004). ADC thresholds were calculated as previously described [10].

Penumbral tissue was assessed retrospectively from ADC-derived lesion growth from 30 minutes and the oedema-corrected infarct volume at 24h. ADC lesion growth at each time point was also expressed as a percentage of final infarct volume to determine the potential impact of intervention at each time point. All analyses were undertaken, blind to the sex of rats.

Statistical analysis

Data are presented as mean ± SD. Infarct volume, ADC threshold values were compared between males and females using a Student's unpaired t-test. Changes in ADC lesion volume and penumbra over time and between sexes were assessed by a two-way ANOVA (between-subjects factor: sex; within-subjects factor: time) with Bonferroni's post-test. $P<0.05$ was considered statistically significant and statistical tests performed using GraphPad Prism v6.

Results

Mortality and physiological parameters

Two of twelve female rats were excluded from analysis due to incomplete MCA occlusion. Three of twelve male rats died overnight before T_2- weighted images could be acquired. Physiological variables remained within normal levels during stroke surgery and throughout MRI scanning and were comparable between the sexes.

Temporal evolution of ADC lesion and final infarct

Oedema-corrected infarct volume at 24hr post-MCAO was significantly larger in males (males 217±49 mm^3, females 151±56mm^3; $P<0.05$). Brain swelling was not significantly different between groups (males: 18.2±3.0%, females: 17.3±1.9% increase of ipsilateral hemisphere, $P=0.3$).

Brain volume at 4h post-MCAO calculated across eight coronal slices, was not statistically different between sexes (males: 901±38mm^3, females: 892±35mm^3, $P=0.6$). Similarly, there were no differences in brain volume at 24h post MCAO between sexes (males: 976±45mm^3, females: 951±33mm^3). There was no evidence of brain swelling as hemisphere volume did

not change significantly over the 0-4h time course. Mean contralateral ADC values were similar between the sexes (males: 0.78±0.02, females: 0.77±0.02 x 10^{-3} mm^2/s, P=0.7) and did not change significantly throughout the 4h scanning protocol. The absolute ADC threshold of abnormality for male rats was 0.58±0.05 x10^{-3} mm^2/s, a 25 ± 2% reduction from mean contralateral ADC values at 4h post-MCAO. In female rats the equivalent ADC threshold was 0.53± 0.03 x10^{-3} mm^2/s, a 29±2% reduction from mean contralateral ADC values. Both the absolute and relative ADC thresholds were similar between the sexes (P>0.05).

From the two-way ANOVA the main effect of sex was significant (P=0.0037, F (1, 19) = 10.97) as was the main effect of time (P<0.0001, F (4, 76) = 112.1). The interaction between the two factors was not significant (P=0.3757). Using the ADC thresholds for each sex, the ADC derived lesions increased significantly over time in males (from 98±64mm^3 at 30min to 223±50mm^3 at 4h; P<0.001) and females (45±28mm^3 at 30min to 147±50mm^3; P<0.001). ADC lesions were significantly smaller in female rats from as early as 60min post-MCAO and this effect was maintained throughout the 4h time course (Figure 1A).

Temporal change of the ischaemic penumbra

Penumbral tissue was assessed retrospectively from the growth of the ADC lesion into the final infarct at 24h post-stroke. Figure 2 illustrates the growth of the ADC lesion at each time point in the median animal. The absolute volume of penumbra determined by comparing the ADC lesion at each time point with 24h infarct decreased significantly over time in both male (from 121 ± 41mm^3 at 0.5hr to 0 ± 12mm^3 at 4hr, P<0.0001) and female rats (from 105 ± 44mm^3 at 0.5hr to 4 ± 14mm^3 at 4hr, P<0.0001) with no significant differences between sexes (F (1, 17) = 0.1312, P=0.7216, Figure 1B). Figure 1C shows the amount of penumbral tissue at each time point expressed as a percentage of the final infarct in males and females reflecting the potential impact of intervention at these time points. For instance, at 30min following MCAO, 59±23% of the final infarct in males was potentially salvageable while in females this equated to 70±13% (Figure 1C).

Discussion

The STAIR recommendations highlight the importance of investigating sex differences in experimental stroke studies [11]. It is well established that sex differences exist in relation to infarct size following experimental stroke with females exhibiting smaller infarcts and improved behavioural outcome compared to males [8]. However, to our knowledge, sex differences in the amount of salvageable penumbra and its lifespan have not been studied. This is the first study to (1) define sex-specific diffusion viability thresholds and (2) investigate if sex influences the acute evolution of ischaemic damage, penumbra volume and its loss during the critical first hours following stroke. The major findings are: (1) Calculated diffusion thresholds of tissue abnormality were comparable between the sexes. (2) The acute ADC lesion volume and final infarct were smaller in female rats from as early as 60 minutes post-stroke. (3) Penumbra volume, and its loss over time, was not significantly different between sexes.

(1) Our ADC thresholds of 0.58 and $0.53x10^{-3}mm^2/sec$ (25% & 29% reduction) for male and female rats, respectively, were similar to previously published thresholds for male Sprague Dawley rats [12-14] and co-morbid strains such as the spontaneously hypertensive stroke prone (SHRSP) rat (21% reduction) and its normotensive control the WKY (23% reduction) [10, 15]. This suggests that rat strain and sex do not have a significant influence on the ADC threshold following MCAO.

Using these ADC thresholds, although the absolute ADC lesion volume was smaller in females, the growth in ADC lesion volume (and consequent loss of penumbra over time) was similar in males and females. Previous studies have established sex-specific differences in infarct volume following MCAO with female sex hormones, oestrogen and progesterone having neuroprotective influences [8, 16]. Our data show that ischaemic damage was smaller in female rats from as early as 30 min after MCAO and this was maintained throughout the acute scanning protocol and similarly reflected in the final infarct at 24h.

Retrospective assessment of penumbra volume revealed no significant sex difference in the absolute volume of penumbra nor in loss of penumbra to the ischaemic core suggesting that the therapeutic time window for intervention is similar. However, absolute volumes do not inform on potential impact of an intervention on final outcome, given that final infarct volumes are different. Therefore we calculated penumbra volume as a percentage of the respective final infarct. Within the first hour of stroke onset, an intervention (thrombolysis to

induce reperfusion) could potentially have greater benefit in females in terms of the reduction in final infarct. As a comparison our previous data from normotensive WKY and stroke-prone spontaneously hypertensive rats (SHRSP) demonstrated that early intervention in SHRSP has much less of an impact on the final infarct volume compared to other strains. One limitation of the present study was that we did not know which stage of the oestrus cycle female rats were within at the time of stroke surgery and this may account for increased variability in this group however a recent meta-analysis challenged this assumption [6].

In conclusion, although the amount of brain damage is significantly smaller in females during the acute phase following stroke, there was no difference in the ADC viability threshold, or the amount or lifespan of potentially salvageable penumbral tissue.

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Authors Contribution Statement:

Dr Tracey Baskerville: Contributed to the execution of experiments, MRI scanning, data analysis and preparation of manuscript.

Professor I Mhairi Macrae: Contributed to the writing and editing of manuscript, experimental design and interpretation of data

Dr William M Holmes: Developed the MRI sequences and contributed to editing of manuscript

Dr Christopher McCabe: Contributed to the execution of experiments, MRI scanning, data analysis, preparation of manuscript and experimental design.

Disclosure/Conflict of interest

None

Supplementary information is available at the *Journal of Cerebral Blood Flow & Metabolism* website – www.nature.com/jcbfm

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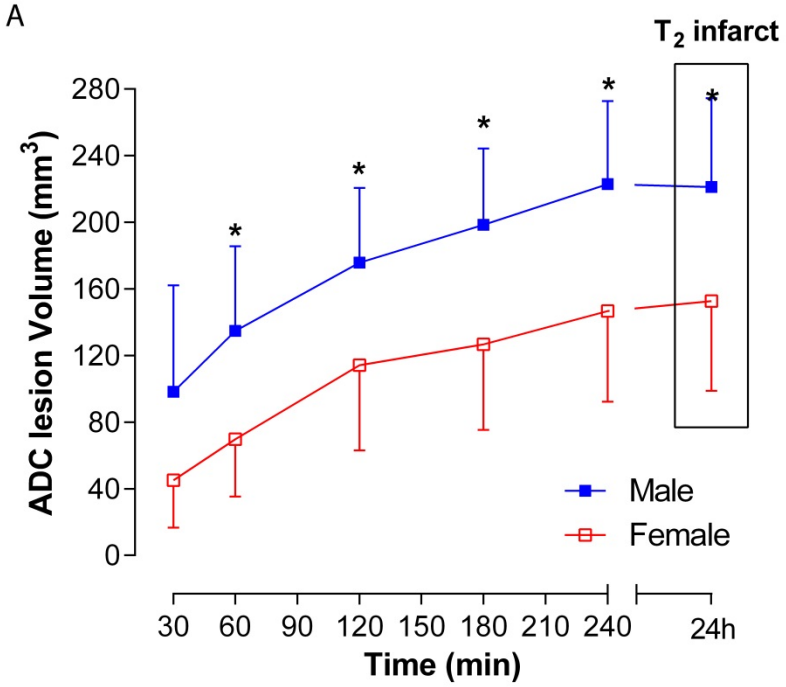
Figure legends

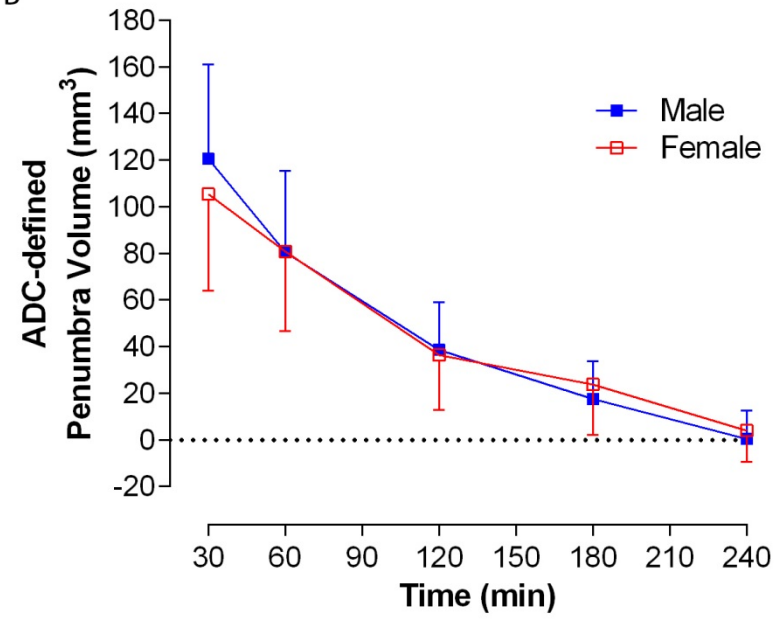
Figure 1. Acute evolution of (A) ADC-defined ischaemic injury and T_2- derived final infarct volume and (B) penumbra volume as defined by ADC lesion expansion into final infarct in male and female rats. (C) illustrates at each time point the amount of penumbral tissue expressed as a % of the respective final infarct in male & female SD rats and for comparison WKY and SHRSP rats (modified from [15]. (*P<0.05, Two way ANOVA, n=10 for both groups). Data are displayed as mean±SD.

Figure 2. Images from the median rat from each group (A: Male; B: Female) showing the spatial location of ADC lesion growth (in white) from 0.5-1, 1-2, 2-3 & 3-4 hours following MCAO. The T_2-weighted infarct (hyperintense region outlined in red) at 24h post- MCAO is shown on the bottom row.

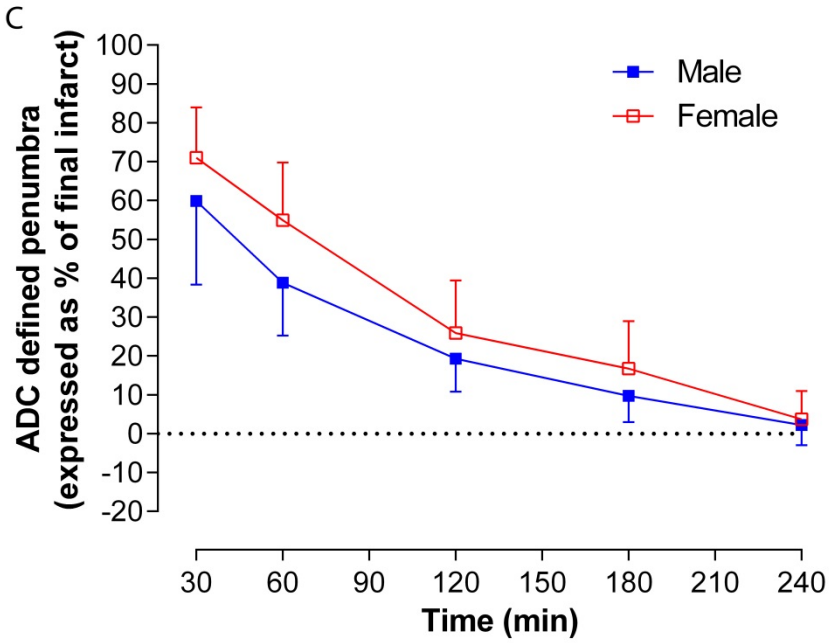
A

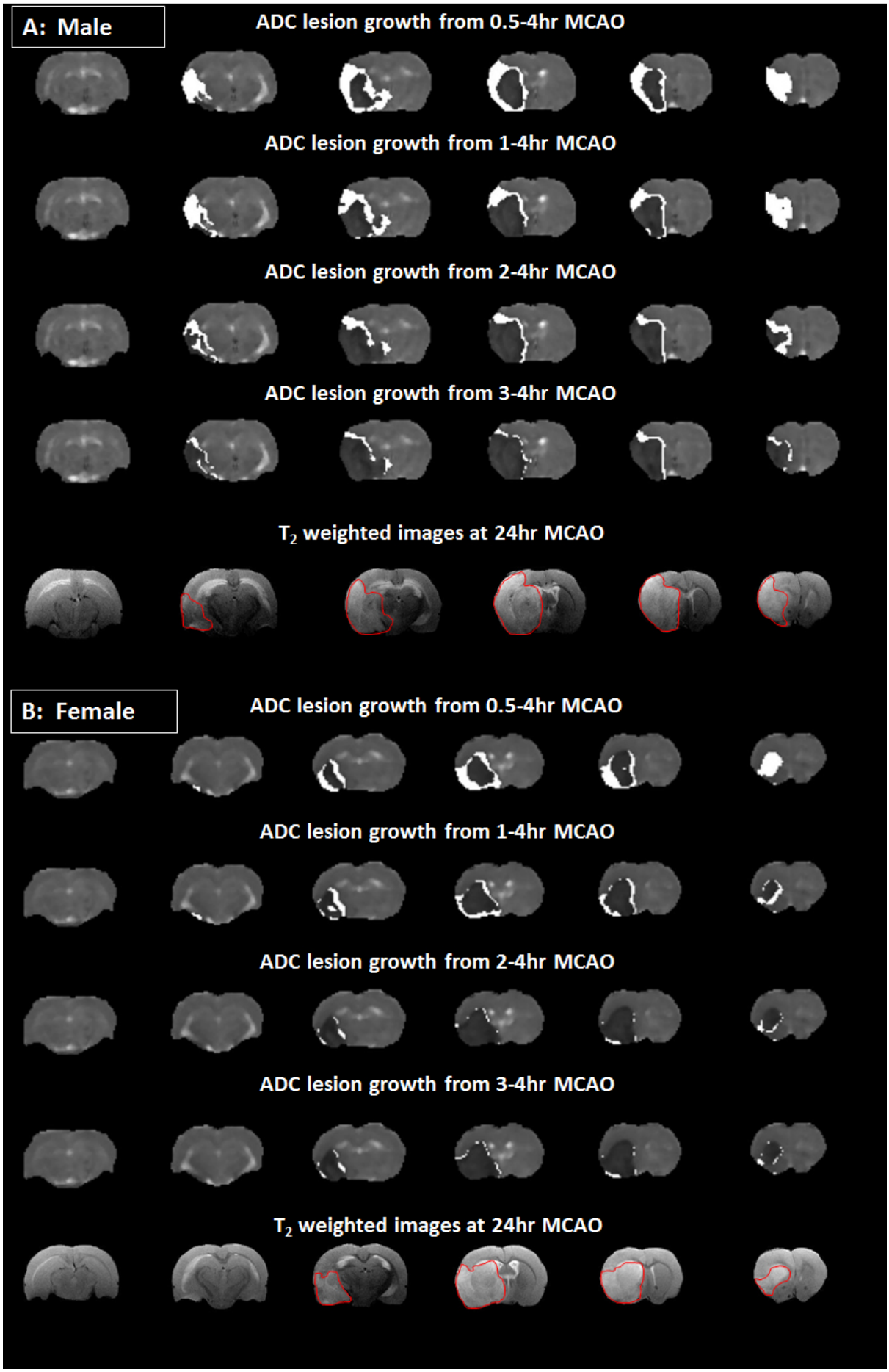

T2 infarct
ADC lesion Volume (mm3)
280
240
200
160
120
80
40
0
*
*
*
*
*
Male
Female
30
60
90
120
150
180
210
240
24h
Time (min)

B


ADC-defined Penumbra Volume (mm3)
180
160
140
120
100
80
60
40
20
0
-20
Male
Female
30
60
90
120
150
180
210
240
Time (min)

C


ADC defined penumbra
(expressed as % of final infarct)
100
90
80
70
60
50
40
30
20
10
0
-10
-20
Male
Female
30
60
90
120
150
180
210
240
Time (min)


A: Male
ADC lesion growth from 0.5-4hr MCAO
ADC lesion growth from 1-4hr MCAO
ADC lesion growth from 2-4hr MCAO
ADC lesion growth from 3-4hr MCAO
T2 weighted images at 24hr MCAO
B: Female
ADC lesion growth from 0.5-4hr MCAO
ADC lesion growth from 1-4hr MCAO
ADC lesion growth from 2-4hr MCAO
ADC lesion growth from 3-4hr MCAO
T2 weighted images at 24hr MCAO